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How do membrane proteins sense water stress?

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Summary

Maintenance of cell turgor is a prerequisite for almost any form of life as it provides a mechanical force for the expansion of the cell envelope. As changes in extracellular osmolality will have similar physicochemical effects on cells from all biological kingdoms, the responses to osmotic stress may be alike in all organisms. The primary response of bacteria to osmotic upshifts involves the activation of transporters, to effect the rapid accumulation of osmoprotectants, and sensor kinases, to increase the transport and/or biosynthetic capacity for these solutes. Upon osmotic downshift, the excess of cytoplasmic solutes is released via mechanosensitive channel proteins. A number of breakthroughs in the last one or two years have led to tremendous advances in our understanding of the molecular mechanisms of osmosensing in bacteria. The possible mechanisms of osmosensing, and the actual evidence for a particular mechanism, are presented for well studied, osmoregulated transport systems, sensor kinases and mechanosensitive channel proteins. The emerging picture is that intracellular ionic solutes (or ionic strength) serve as a signal for the activation of the upshift-activated transporters and sensor kinases. For at least one system, there is strong evidence that the signal is transduced to the protein complex via alterations in the protein–lipid interactions rather than direct sensing of ion concentration or ionic strength by the proteins. The osmotic downshift-activated mechanosensitive channels, on the other hand, sense tension in the membrane but other factors such as hydration state of the protein may affect the equilibrium between open and closed states of the proteins.

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Introduction

Enzymes and other macromolecules are not only sensitive to physical parameters such as pH, temperature and solute composition but also to water activity. The majority of microbial cells experience changes in extracellular water activity, which has direct consequences for the water activity of the cytoplasm. Following an increase in external water activity (osmotic downshift), passive influx of water will increase the turgor and eventually lyse the cells if there are no mechanisms to counteract the stress. Similarly, upon osmotic upshift, water will flow out of the cell, the turgor will decrease and, in the end, the cells will plasmolyse. To keep turgor within a specific range, and to prevent cells from lysing or plasmolysing, microbes adjust their intracellular osmolyte concentrations. Both Gram-positive and Gram-negative bacteria prefer particular zwitterionic organic cosolvents such as glycine betaine, carnitine or ectoine as osmoprotectants (Wood, 1999). These compounds, generally referred to as compatible solutes, can be accumulated to molar levels without negative effects on macromolecular structure or function. In fact, several compatible solutes have been shown to stabilize enzyme structure (Arakawa and Timasheff, 1985). The stabilization of native protein structures by these compounds involves preferential exclusion of the compatible solutes from the protein's surface. The preferential exclusion implies that the interaction between protein and compatible solutes is thermodynamically unfavourable. Because more protein surface is exposed in the unfolded than in the native state, the free energy difference for the transfer from water to compatible solute solution is largest for unfolded protein. This large positive Gibbs energy effect renders proteins thermodynamically more stable in the presence of compatible solutes.

Because the *de novo* synthesis of additional transporters and biosynthetic enzymes would be too slow to be effective against a rapid external osmotic change, cells need transport systems for compatible solutes that are directly controlled by osmotic conditions. To accumulate

compatible solutes upon osmotic upshift, bacteria use ATP-binding cassette (ABC) or ion-motive-force driven transporters. Structurally, these transporters are not readily discriminated from other, non-osmoregulated members of the transporter family to which they belong. To excrete compatible solutes, that is, in the event the turgor becomes too high, organisms activate mechanosensitive channels. The molecular identities of three proteins that contribute to these channel activities have been identified (Sukharev *et al*., 1994; Levina *et al*., 1999). Genomic sequencing of several bacterial species demonstrates that homologues of one or more of these molecules are present in many diverse bacterial species (Levina *et al*., 1999; Maurer *et al*., 2000). By far, the best studied of the three is the protein responsible for the largest conducting activity, MscL, for which a large number of mutants from *Escherichia coli* and a crystal structure of the protein from *Mycobacterium tuberculosis* are available; (Chang *et al*., 1998; Ou *et al*., 1998). Combining mutational and structural data has recently led to models for channel gating (Blount and Moe, 1999; Sukharev *et al*., 2001a; b). Upon gating, MscL jettisons solutes with little discrimination, except for size. Apparently even small proteins, which are detected in the medium subsequent to osmotic downshock, can leave the cell through this channel (Ajouz *et al*., 1998). MscL, with its huge pore (conductance of 3.6 nS and estimated pore of 30–40 Å in diameter) appears to be a final resort to release high pressures resulting from acute external osmotic downshifts. Homologues from several species have been shown to encode channel activity when expressed *in trans* in *E. coli*, strongly suggesting that they are orthologues (Moe *et al*., 1998). Interestingly, at least one marine bacterial species, *Vibrio alginolyticus*, does not appear to contain a MscL and is sensitive to osmotic downshift, whereas expression of the *E. coli* MscL *in trans* allows this cell to survive such a challenge (Nakamaru *et al*., 1999). The reduction of osmotic pressure of the cytoplasm at less severe hypoosmotic stresses seems to be mediated by other mechanosensitive channels (e.g. MscS in *E. coli*, which is more sensitive to membrane tension and only 1 nS in conductance) and the activation of solute-specific efflux systems (Poolman and Glaasker, 1998; Levina *et al*., 1999).

This review addresses the osmosensing mechanisms of the transporter and channel molecules that enable bacteria (and archaea) to respond rapidly to osmotic shifts. The osmotic downshift–activation of MscL (Blount and Moe, 1999) is compared with the mechanism(s) of osmotic upshift–activation of the ABC transporter OpuA from *Lactococcus lactis* (Obis *et al*., 1999; van der Heide and Poolman, 2000a; van der Heide *et al*., 2001), and the ion-linked transporters BetP from *Corynebacterium glutamicum* (Rübenhagen *et al*., 2000; 2001) and ProP from *E. coli* (Racher *et al*., 1999; 2001) as well as osmosensing by signal transduction pathways involved in the regulation of osmotically controlled genes, e.g. the KdpD and EnvZ sensor kinases from *E. coli* (Jung *et al*., 2000; 2001) (Table 1). Each of these systems has been studied not only *in vivo,* but also in proteoliposomes, thus allowing detailed analysis of the osmosensing mechanisms. The *in vitro* studies provide the basis for the proposal of some unifying mechanisms of osmosensing, which are supported by *in vivo* data. For general aspects of osmoregu-

Table 1. Properties of osmotic up- and downshift-activated membrane proteins.

Name	Type of system	Function	Other features
BetP	Δs-Driven transporter	Uptake of glycine betaine	Activated by osmotic upshift; sensing of K^+
EnvZ	Sensor kinase of two-component regulatory system	Regulation of expression of outer membrane porins C and F	Activated by osmotic upshift; sensing of ionic osmolytes
KdpD	Sensor kinase of two-component regulatory system	Regulation of expression of P-type K^+-ATPase	Activated by osmotic upshift; sensing of ionic osmolytes; additional specific control via K^+ sensing
KefA	Mechanosensitive channel	Efflux of osmolytes	Activated by osmotic downshift; minor component of MscS activity; sensing of tension in the membrane
MscL	Mechanosensitive channel	Efflux of osmolytes	Activated by osmotic downshift; sensing of tension in the membrane
OpuA	ATP-binding Cassette (ABC)-transporter	Uptake of glycine betaine	Activated by osmotic upshift; sensing of intracellular ionic osmolytes via changes in protein–lipid interactions
ProP	Δp-Driven transporter	Uptake of proline	Activated by osmotic upshift
YggB	Mechanosensitive channel	Efflux of osmolytes	Activated by osmotic downshift; major component of MscS activity; sensing of tension in themembrane

Δs and Δp refer to sodium- and proton motive force, respectively.

lation and mechanosensation in bacteria, we refer the reader to previous reviews by Booth and Louis (1999), overview of how a cell manages hypoosmotic stress; Csonka and Hanson (1991), possible mechanisms underlying osmoregulatory responses; Hamill and Martinac (2001), molecular basis of mechanosensation; Poolman and Glaasker (1998), overview of how a cell regulates pool sizes for compatible solutes; and Wood (1999), most comprehensive and recent review on osmoregulation in bacteria.

Mechanisms of osmosensing

Osmotic activation of membrane proteins may be signalled via: (i) a change in cell turgor; (ii) mechanical deformation of the membrane (macroscopic change in membrane structure); (iii) mechanical stimulus originating within the exo- or cytoskeleton of the cell; (iv) a change in the hydration state of the protein (internal or external osmolality); (v) alterations in the physicochemistry of the membrane bilayer (protein–lipid interactions); (vi) a change in cytoplasmic ion concentration or ionic strength; and (vii) specific molecules interacting directly with the protein. With regard to the physicochemistry of the membrane, a number of properties are affected as a function of osmotic shift, including membrane fluidity, bilayer thickness, hydration state of lipid headgroups, and interfacial polarity and charge. Each of these parameters contribute to the lateral pressure of a membrane. The lateral pressure is composed of the components of the interactions between the various membrane constituents, specifically the derivative of their free energy with respect to area. The lateral pressure profile is the depth-dependent distribution of lateral stresses within the membrane (Cantor, 1999), a global parameter that is often discussed in terms of 'intrinsic strain', 'intrinsic curvature', 'curvature strain' or 'internal tension' (Gruner, 1985; Marsh, 1996; Wood, 1999). In this paper, we use lateral pressure profile as description of the lateral stresses in lipid membranes. The relevance of each of the physicochemical parameters of the membrane to osmosensing and the available evidence for a particular mechanism are described in the following sections.

Cell turgor

Cell turgor (ΔP) is the hydrostatic pressure difference that balances the difference in internal and external osmolyte concentration. In the equation:

$$\Delta P = (RT/V_w)\ln(a_o/a_i) \approx RT(c_i - c_o)$$

in which V_w is the partial molal volume of water, 'a' is the water activity, 'c' is the total osmolyte concentration, and the subscripts 'i' and 'o' refer inside and outside respectively. A cell plasmolyses when ΔP becomes negative. Although cell turgor is required for the expansion of the cell wall, there is little information on what the lower limit of turgor should be before cell growth ceases. Depending upon the species, a bacterial cell may develop several to a few tens of atmospheres of pressure across the cell envelope. For a given organism, cell turgor will vary in response to changes in external osmolality. In *E. coli*, the turgor decreases from ≈3 to 0.5 atm when the osmolality of the growth medium is increased from 0.03 to 0.8 osm kg^{-1} (Cayley *et al*., 2000). Although a turgor of 0.5 atm may be sufficient to sustain growth of *E. coli*, it is quite possible that the lower limit of turgor of Gram-positive bacteria, with their generally thicker cell wall, is much higher. In theory, osmosensors could detect changes in cell turgor. In fact, such a model has been proposed for the membrane-bound sensor kinase KdpD, a signal transduction component that osmotically regulates expression of the *E. coli*, *kdpFABC* P-type potassium-ATPase operon (Laimins *et al*., 1981). This model was rooted in the assumption that turgor is maintained across the cytoplasmic membrane. However, there is evidence that in Gram-negative bacteria, the cytoplasm and periplasm are isotonic and that turgor is maintained across the cell wall–outer membrane complex rather than cytoplasmic membrane (Cayley *et al*., 2000). If true, turgor would probably not be the primary regulatory signals for transporters or signal transduction components present in the cytoplasmic membrane. Finally, as membrane vesicles and liposomal systems can only withstand low pressures as compared with cells with a peptidoglycan layer (Csonka and Hanson, 1991), functional incorporation into such artificial membranes of sensors that respond to low turgors should lead to constitutive activity. Several classes of osmoregulated systems, however, including the ABC transporter OpuA from *L. lactis* (van der Heide and Poolman, 2000a), the ion-linked transporters BetP from *C. glutamicum* (Rübenhagen *et al*., 2000), ProP from *E. coli* (Racher *et al*., 1999), and the sensor kinases KdpD and EnvZ from *E. coli* (Jung *et al*., 2000; 2001) show normal functional regulation when present in membrane vesicles or incorporated into proteoliposomes, suggesting that turgor is not the salient stimulus. Together, these findings suggest that it is unlikely that any bacterial osmo-sensing cytoplasmic membrane molecules respond directly to changes in cell turgor.

The issue of whether mechanosensitive channels respond to pressure across the membrane or tension within it has been answered directly. One can determine if a channel is sensing membrane pressure or tension by imaging the curvature of a membrane patch, measuring the pressure across the membrane, and using Laplace's law:

$$t = p * r/2$$

in which 't', 'p' and 'r' refer to tension, pressure and radius of curvature respectively. Such analysis clearly demonstrated that MscL senses tension within the membrane, not the pressure across it (Sukharev *et al*., 1999). Although the pressure required to gate the MscS channel has been observed to vary from patch to patch, it is always two-thirds of that required to gate MscL (Blount *et al*., 1999), strongly suggesting that this channel also responds exclusively to membrane tension. Finally, by measuring whole-cell currents in yeast of different size, it has been demonstrated that mechanosensitive channels in this system are also gated by tension, suggesting this mechanism may be conserved among eukaryotes as well (Gustin *et al*., 1988). Hence, even for mechanosensitive channels it appears that these sensors do not sense cell turgor directly.

Mechanical deformation of the lipid bilayer

On the assumption that the lipid bilayer behaves as an elastic solid, the intrinsic mechanical properties of the membrane can be described by four elasticity moduli that describe the response of a unit area of bilayer to volume compression, area expansion, bending/curvature and extension/shear (Evans and Skalak, 1980). The response of membranes to these elastic deformations has been recently reviewed by Hamill and Martinac (2001), and their main conclusions relevant for this paper are summarized here. First, the bilayer is at least 10-fold more compressible in area than in volume during mechanical deformations encountered under physiological conditions. Osmotic downshifts will thus lead to relative increases in membrane area and concomitant decreases in membrane thickness. Second, the bending rigidity of the bilayer is dependent upon the lipid composition and area of each monolayer, and this parameter determines, amongst others, the shape of lipid vesicles. Third, above the phase transition temperature, the membrane behaves like a fluid in response to extension. Of the elastic deformations, the ones that lead to thinning of the membrane are thought to have major impact on protein conformations and may thus signal activity changes. For the mechanosensitive channel MscL, it is thought that thinning of the membrane upon osmotic downshifts contributes to the ability of the protein to sense membrane tension (Hamill and Martinac, 2001).

As proteoliposomes behave osmometrically, that is, water diffuses across the membrane in response to the osmotic difference between the inner compartment and the outside medium, they are expected to decrease their volume to surface ratio when the outside osmolality is increased. The changes in membrane structure and lumen contents (osmolyte concentration) in osmotically

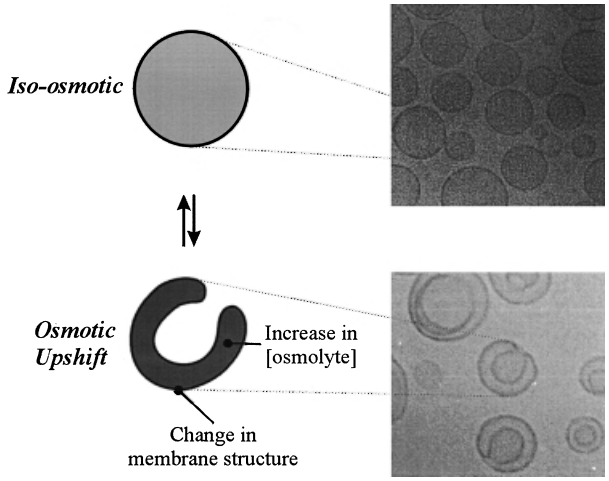

Fig. 1. Macromolecular structure of proteoliposomes subjected to osmotic upshift. Schematic representation and cryo-electron microscopic images of proteoliposomes prepared under iso- and hyperosmotic conditions using membrane impermeant osmolytes. Hyperosmotic conditions were effected by the addition of 200 mM KCl to a medium of 190 mosmol kg^{-1}, resulting in a final osmolality of 535 mosmol kg^{-1}. For details, see van der Heide *et al.* (2001).

stressed proteoliposomes may be compared with those in cells that are in a state of plasmolysis (Woldringh, 1994). Proteoliposomes with an average diameter of 200 nm change their shape from spherical to sickle-shaped upon osmotic upshift (van der Heide *et al*., 2001) (Fig. 1). These morphological changes occur within the millisecond-to-second time range. However, for the ABC transporter OpuA and the sodium motive force-driven glycine betaine transporter BetP, which remain the only transporters rigorously investigated, it has been clearly shown that these macroscopic deformations of the membrane are not critical for osmosensing and regulation of the systems (van der Heide *et al*., 2001; Rübenhagen *et al*., 2001). Hence, in contrast to the osmotic downshift-activated mechanosensitive channel proteins, there is currently no evidence that osmotic upshift-activated systems are sensitive to 'physiologically relevant' mechanical deformations of the membrane.

Mechanical deformation of proteins

In eukaryotic cells, the cytoskeleton and extracellular matrix affect the function of some membrane transport proteins. For at least one family of eukaryotic mechanosensitive channels, interactions with intra- and extracellular proteins are postulated to play an active role in channel gating (Tavernarakis and Driscoll, 1997). Traditionally, it was thought that bacteria lack a cytoskeletal structure but compelling evidence for a bacterial protein that assembles into F-actin-like filaments has been obtained recently (van den Ent *et al*., 2001). Homologues of this protein, MreB, are widely distributed among rod-

shaped, filamentous and helical bacteria, suggesting that the MreB skeleton is important for the non-spherical shape of these organisms (Jones *et al*., 2001). In addition to this cytoskeleton, most bacteria also have a peptidoglycan exoskeleton or cell wall. This scaffolding could serve a specific role in the regulation of membrane protein function. However, at least for the bacterial mechanosensitive channel MscL and the osmotically regulated transport molecules BetP, OpuA and ProP, no auxiliary protein is required for function. Insertion of pure protein into synthetic lipids is all that is necessary to reconstitute the osmoregulated activity (Blount *et al*., 1996; Racher *et al*., 1999; 2001; van der Heide and Poolman, 2000a; Rübenhagen *et al*., 2000; van der Heide *et al*., 2001). Similar studies suggest the same is true for YggB, which constitutes the major component of the MscS activity observed in *E. coli* (P.C. Moe and P. Blount, unpublished). On the other hand, a mechanosensitive channel, KefA, which contributes a minor component of the *E. coli* MscS activity, contains a large hydrophilic amino terminus that is predicted to be periplasmic. Much larger than its mechanosensitive counterparts, this channel might have functional interactions with the peptidoglycan layer, or even with outer membrane elements. To date, functional reconstitution has not been reported for this channel. Interestingly, this channel is observed in only about 25% of patches when native membranes of giant protoplasts are assayed by patch-clamp (Levina *et al*., 1999); one interpretation is that the channels cluster, perhaps due to cytoskeletal or cell wall interactions. Note that as the radius of curvature increases, less pressure is required to achieve gating tensions (Laplace's law, see above). Hence, localization of these channels to subcellular regions in which the membrane assumes a specific radius of curvature (location-specific in the *E. coli* rod-shaped organism) would effectively set the channels' sensitivity to a specific turgor. Alternatively, auxiliary proteins may be required for gating, or residual cell wall or outer membrane present in the minority of patches could explain this phenomenon. In either event, it appears likely that extra-membranous components directly or indirectly regulate the mechanosensitive channel KefA. An accessory cyto-plasmic protein (ProQ) has been proposed to regulate the proline transporter ProP from *E. coli*, even though ProP on its own seems sufficient and osmotically functional in proteoliposomes (Kunte *et al*., 1999; Racher *et al*., 1999). As mutations in *proQ* do not affect the expressed levels of ProP, the regulation must occur post-translationally but the mechanism by which ProQ acts remains elusive.

Hydration state

Substrate and ligand binding to enzymes and transporters is typically associated with changes in the conformation of the proteins. As it is likely that different protein conformations sequester different amounts of water, osmotic stress could potentially affect a system's activity through a change in the hydration state of the protein. Hexokinase is a classic example of water activity as regulator of enzyme activity (Parsegian *et al*., 1995). The dissociation constant (K_d) for glucose binding to hexokinase decreases with increasing osmotic pressure of the assay medium, when varied with either low or high molecular weight polyethylene glycol (PEG) in the solution (Reid and Rand, 1997). The smaller effects of the low molecular weight PEGs are explained by their less effective steric exclusion from a cleft in the surface of the enzyme (see also hereafter). Similarly for the channel-forming peptide alamethicin, it has been shown that the open probability decreases with increasing concentrations of high molecular weight PEGs (Vodyanoy *et al*., 1993). There is no change in open probability when the water activity is varied with low molecular weight PEGs. Parsegian and colleagues (Parsegian *et al*., 1995) have formulated a thermodynamic hypothesis for these observations. In the transition from the closed to the open state, the channel will require additional water as the open state is most probably more hydrated. Solutes too big to enter the channel, such as high molecular weight PEGs, will compete with the protein for water. Consequently, the excluded solute will cause the channel to perform extra osmotic work, which will lower the probability of the open conformation. The extra amount of work is less with solutes that are able to enter the channel, for example low molecular weight PEGs. The low molecular weight PEGs give rise to a smaller excluded volume and are thermodynamically less unfavourable than high molecular weight PEGs. In other words, the low molecular weight PEGs have a smaller dehydrating effect on the protein than high molecular weight ones.

The effects of osmotic pressure of the bath or pipette solution in patch-clamp experiments with MscL have not been directly studied by using PEGs as outlined above. However, one model, referred to as the 'hydrophobic lock' hypothesis (Chang *et al*., 1998; Blount and Moe, 1999), combines interpretations of the mutagenic data from *E. coli* with the structural model from *M. tuberculosis.* This theory proposes that the major energy barrier to channel opening lies in 'breaking' this lock which rests at the most constricted part of the closed channel pore, transiently exposing these hydrophobic residues to a hydrophilic environment, most likely the water in the lumen of the opening pore (Blount and Moe, 1999; also see below). If true, the dehydration of the pore surface would lead to a decreased energy barrier for channel gating, reflected in rapid channel kinetics as seen with certain mutants (Ou *et al*., 1998; Yoshimura *et al*., 1999); this could oppose

the gating of the channel upon osmotic downshift as the water activity would be higher under those conditions. On the other hand, it seems likely that in the fully open state, similar to the alamethicin channel, MscL would be more hydrated. Although hydration may serve in a modulatory capacity, it is unlikely to have a significant influence on microbial mechanosensitive channel gating, which is effected in patch-clamp by adding tension to the membrane.

In addition to the thermodynamic perspective of osmotic stress, there are other factors one must consider when using high and low molecular weight solutes to study membrane-embedded proteins. Unlike the low molecular weight PEGs, those with a mass above 500 Da are membrane-impermeable (Racher *et al*., 2001). Osmotic upshifts will thus result in 'permanent' water efflux from compartmentalized systems such as proteoliposomes, which will increase the surface to volume ratio of the vesicles and thereby the intraliposomal osmolyte concentration (Fig. 1). In the case of OpuA and BetP, it has been shown that the increase in proteoliposomal cation concentration is sufficient to activate the system (see also below). Whether lumen ion concentration affects the alamethicin channels is not known, but this aspect must be taken into account for a complete understanding of the mechanisms of osmosensing by membrane proteins.

On the basis of the effects of low and high molecular weight PEGs on the osmotic activation of the proton-linked co-transporter ProP from *E. coli*, Racher and colleagues (Racher *et al*., 2001) have proposed that surface hydration of the protein acts as a regulator of the system, either alone or in combination with some correlate of vesicle shrinkage. It has been observed that low molecular weight, membrane-permeable, PEGs activated membrane-reconstituted ProP albeit less efficiently than the membrane impermeable ones. The authors argue that, similar to hexokinase, ProP is more dehydrated and more completely activated with PEGs that are more effectively excluded from the hydrophilic surfaces of the protein. However, as in the situation with the alamethicin channels, alternative explanations have not been rigorously ruled out. The low molecular weight PEGs diffuse relatively slowly across the liposomal membrane, thereby resulting in a transient deformation of the bilayer and concentration of the luminal content (van der Heide *et al*., 2001). As the kinetic resolution of the reported volume measurements is insufficient to observe the transient changes imposed by the permeant osmolytes, the system may have been in the activated state for a short period. If true, the observed activity in the presence of low molecular weight PEGs could have a similar mechanistic basis as the activation by truly membrane-impermeant osmolytes.

Physicochemical properties of the membrane

Detailed experimental information about the role of the membrane in the mechanism of an osmoregulated transporter is only available for OpuA from *L. lactis* (van der Heide and Poolman, 2000a; van der Heide *et al*., 2001), but the published data on BetP from *C. glutamicum* are entirely consistent with the observations made for the ABC transporter (Rübenhagen *et al*., 2000; 2001). To define the osmosensing mechanism of OpuA, the protein was incorporated into liposomes of different lipid composition, thereby varying physicochemical properties of the membrane such as fluidity, bilayer thickness, charge and polarity of lipid headgroups, interfacial polarity and charge, and/or lateral pressure. Osmotic activation profiles, as depicted in Fig. 2, have been determined, which provide information about the threshold osmolality difference required for activation and the maximal activities of the transporter (van der Heide *et al*., 2001). For the interpretation of the data, the assumption was made that variations in the threshold osmolality difference required for activation indicate factors relevant for osmosensing, whereas variations in the actual maximal rates attained do not. The validity of this approach is partly rooted in the

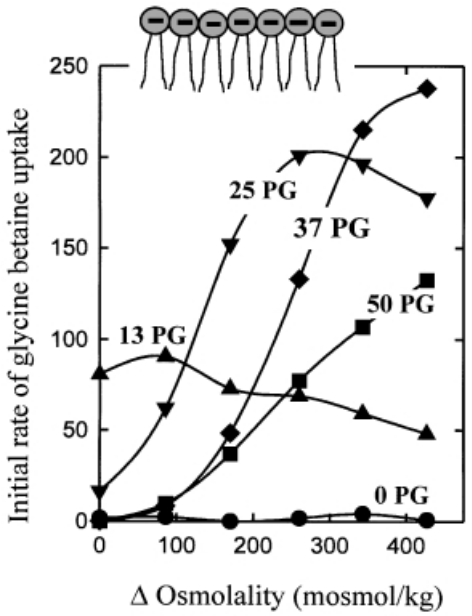

Fig. 2. Osmotic activation profiles of OpuA in proteoliposomes with varying amounts of anionic lipid. The proteoliposomes were composed of 50 mole-% DOPE and 0 (●), 13 (▼), 25 (◆), 37 (○) or 50 mole-% DOPG (▲) plus 50, 37, 25, 12 or 0 mole-% DOPC respectively. The vesicle lumen consisted of an ATP-regenerating system to yield a constant level of ATP for prolonged periods of time. The concentration of Mg^{2+} was kept equal to the total concentration of nucleotides (ATP plus ADP) to minimize possible membrane-perturbing effects of the divalent cation. The osmolality of the medium was varied with KCl. ΔOsmolality refers to the initial difference in external and internal osmolality.

findings that the maximal rates of non-osmoregulated transporters from *L. lactis (*and related organisms) are similarly dependent on lipid composition as OpuA, for example anionic and non-bilayer lipids are critical for activity and optimal activity is reached with C16 or C18 acyl chains (unpublished results on the lactose transport system *Streptococcus thermophilus*; In't Veld, 1992). The fraction of anionic (charged) lipids was found to be of major importance for the osmotic activation of OpuA, whereas variations in acyl chain length, position and configuration (*cis/trans*) of the double bond, and fraction of non-bilayer lipids had relatively minor effects. By varying the fraction of anionic lipids (DOPG or DOPS) from <6–13–25% (Fig. 2), OpuA is converted from an inactive (I) to an 'constitutively' active (C) to an osmotically controllable (R) state. Moreover, at 25 mole-% of DOPG, the OpuA system is converted from R to C by the cationic amphipath tetracaine, whereas the anionic amphipath decanoic acid shifts the equilibrium towards R. These small, charged amphiphilic molecules partition into the lipid bilayer with their most hydrophilic moiety at the phospholipid headgroup level. The measurements in the proteoliposomes with varying lipid (and amphipath) composition show that the overall charge of the headgroup region of the membrane lipids determines the activity (kinetic state) of the transporter. Similar to the observations made for OpuA, the optimum for osmotic stimulation of BetP from *C. glutamicum* shifts to higher values of osmolality with increasing amounts of phosphatidylglycerol lipids in the membrane, pointing towards a role of the membrane in transducing ionic changes to the protein (Rübenhagen *et al*., 2000).

In the context of membrane protein function in relation to osmotic stress, the role of bilayer thickness and lateral pressure require further discussion. Bilayer thickness is obviously important for any membrane protein, as mismatch would result in exposure of hydrophobic surfaces of either the protein or lipid to an aqueous environment. The membrane–water interface of the bilayer comprises a chemically complex environment, which offers multiple possibilities for interactions with protein side-chains (Killian and von Heijne, 2000). If the bilayer thickness is suboptimal for these interactions, the protein or lipid may react to prevent hydrophobic mismatch that may lead to alterations in protein conformation and activity. When OpuA was incorporated in membranes in which 50% of the lipid fraction was varied in terms of acyl chain length (from C14 to C22), the protein displayed a clear optimum in maximal activity at C18 (dioleoyl lipids), but the threshold for osmotic activation was the same in all cases (van der Heide *et al*., 2001). Thus, changes in bilayer thickness upon osmotic up- or downshift are not of prime importance for the regulation of OpuA activity. On the other hand, in one model for gating the mechanosensitive channel MscL, the tilts of the transmembrane α-helices are predicted to increase as they move away from the axis of the pore, that is, when the membrane expands (Sukharev *et al*., 2001a; b). The osmotic downshift-induced thinning of the membrane and the resulting hydrophobic mismatch could thus provide at least part of the energy required for the transit from the closed to the open state. If the postulate is true, one would expect that the membrane tension required for MscL gating would increase with increasing bilayer thickness as proposed by Hamill and Martinac (2001).

The different local intermolecular forces between lipid molecules in a fluid membrane originate from steric hindrance, hydration, electrostatic charge and/or hydrogen bonding in the headgroup region, interfacial tension and acyl chain pressure. The differences in the components of the interactions as a function of membrane depth lead to enormous local transverse pressures that correspond to bulk pressures of several hundreds of atmospheres (Marsh, 1996; Cantor, 1999). Thus the local pressure as a function of membrane depth is non-uniform; this parameter is referred to as the lateral pressure profile. Statistical thermodynamic calculations of the equilibrium pressure profiles of membranes predict large redistributions of lateral pressure when the acyl chain length, the degree, position and configuration of unsaturation, or headgroup repulsion are varied (Cantor, 1999). Similarly, the incorporation into a lipid membrane of cholesterol or interfacial active solutes such as anaesthetics are predicted to increase the lateral pressure selectively near the aqueous interfaces, resulting in a compensating decrease in lateral pressure near the centre of the bilayer. Such changes in the lateral pressure profile have been postulated to influence protein conformation and activity (Cantor, 1999). Because the osmotic activation profile of the OpuA transporter of *L. lactis* is not affected by variations in acyl chain length, position and configuration of the unsaturation, and/or headgroup repulsion as long as the charge of the lipid headgroups is kept constant (van der Heide *et al*., 2001), it seems unlikely that osmotic stress is detected as a perturbation of the lateral pressure profile. Although supported by a less elaborate set of experiments, a similar conclusion can be reached for the BetP glycine betaine transporter of *C. glutamicum* (Rübenhagen *et al*., 2001). Again, in contrast with these osmotic upshift-activated transporters, the opening of the mechanosensitive channel MscL may be linked directly to the overall decrease in lateral pressure that one would predict when the membrane expands following osmotic downshift. System responses to changes in the lateral pressure profile and bilayer thickness are not readily dissected as these membrane parameters are inherently

related. However, by calculating the equilibrium properties of mixed lipid systems, Cantor (1999) has proposed lipid compositions having very different lateral pressure profiles but a constant membrane thickness. It would be interesting to determine the membrane tension dependence of MscL in such lipid mixtures, but one should be aware that certain lipid compositions may lead to phase separation, heterogeneity in the membrane that is not readily predicted from calculations of the lateral pressure profiles by molecular dynamics simulations. MscL may also hydrogen bond with specific lipid headgroups, as has been predicted by dynamic simulations (Elmore and Dougherty, 2001). While it is likely that the influences of membrane thickness (hydrophobic mismatch), lateral pressures and protein–lipid interactions are key stimuli for microbial mechanosensitive channel gating, a more systematic approach is necessary for their dissection.

Ionic osmolytes and ionic strength

By varying the concentration and composition of osmolytes at the cytoplasmic face of the OpuA protein, it has been shown that only ionic osmolytes activate the system (van der Heide *et al*., 2001). These studies eliminate water activity or a specific signalling molecule as signals for osmotic activation. As the shifts in the activation profile of OpuA by ionic osmolytes are similar to those effected by alterations in the fraction of anionic phospholipids or the insertion of charged amphiphiles into the membrane, the data strongly suggest that OpuA senses osmotic stress via perturbations in the ionic interactions between protein and bilayer lipids. Rübenhagen and colleagues (Rübenhagen *et al*., 2001) have concluded that BetP from *C. glutamicum* is a sensor for cytoplasmic potassium because K^+ (and the analogues Rb^+ and Cs^+) activate the system, whereas organic cations and NH_4^+ do not. Unfortunately, the authors have not been able to test Na^+ and Li^+, which leaves the possibility open that BetP, too, is actually sensing inorganic cations rather than specifically K^+. Osmotic activation of the membrane-bound histidine kinase KdpD of the *kdpFABC* operon in *E. coli* may similarly involve sensing of cytoplasmic ion concentration or ionic strength, but this system has additional, specific, mechanisms to sense K^+ (Sugiura *et al*., 1994; Jung *et al*., 2000). The sensing of cytoplasmic ion concentration by KdpD can be observed in a set of mutants insensitive to the K^+ signal, as discussed in the next section. The sensor kinase EnvZ, reconstituted in proteoliposomes, is activated by K^+, Na^+, Rb^+ and NH_4^+, irrespective of the counterion (Cl^-, Br^-, SO_4^{2-}, NO_3^- or glutamate), whereas neutral osmolytes have no effect (Jung *et al*., 2001). Although the highest stimulation of autokinase activity was achieved with K^+ as the cation, the data are in agreement with the model in which the cytoplasmic face of EnvZ senses the concentrations of ionic osmolytes. Whether the ionic signal is transduced to the proteins via the membrane has not been established for the sensor kinases but there is evidence to support such a mechanism. Cationic amphipaths such as chlorpromazine and procaine can function as effectors of KdpD, that is, the compounds induce the expression of the *kdpFABC* operon when the appropriate mutants are tested (Sugiura *et al*., 1994). Moreover, the autophosphorylation activity of KdpD is dependent on negatively charged phospholipids (Stalkamp *et al*., 1999).

With regard to the stimuli for the sensor kinases, there is some controversy in the literature about the activation by ionic and non-ionic osmolytes (e.g. are ionic osmolytes more efficient?), the influence of amphipathic compounds (is the signal mediated via the membrane?), the equivalence of K^+ and Na^+ as inorganic cation, and so forth. In the case of KdpD, the dual regulation by K^+ and ionic strength is an additional complicating factor. We feel that the regulation of the sensor kinases can be largely rationalized within the 'ionic strength model', and that apparent discrepancies are due to flaws in the design of the experiments. For instance, some of the autophosphorylation assays for the sensor kinases have been performed with the ionic ('activating') and non-ionic ('non-activating') osmolytes having direct access to the phosphorylation domain of the protein, that is, in inside-out membrane vesicles or proteoliposomes with inside-out reconstituted protein (Jung *et al*., 2001). If ionic strength is sensed at the cytoplasmic face of the EnvZ protein, one would expect activation by ionic and not by non-ionic osmolytes, which is the actual observation. On the other hand, one expects activation of the sensor kinase by neutral membrane-impermeable osmolytes when the compounds are added to a compartmentalized system (cell or proteoliposome) in which the protein has the kinase site on the inside. The activation by neutral osmolytes would be indirect, that is, through the increase in concentration of internal ionic osmolytes. Jung and colleagues (Jung *et al*., 2000; 2001) have also studied kinase activation in right-side-out membrane vesicles, that is, with the phosphorylation domain on the inside. To allow ATP to enter the membrane vesicles, the experiments were performed in the presence of 20 mM $MgCl_2$. The high concentration of magnesium, however, causes restructuring of the membrane and membrane permeabilization. Consequently, addition of ionic or non-ionic osmolytes will not result in vesicle shrinkage and the accompanying increase in the concentration of lumen contents as observed in tightly sealed proteoliposomes (see Fig. 1). The added ionic and non-ionic osmolytes will permeate the membrane and thereby reach the phosphorylation domain of KdpD and EnvZ. Depending on the permeability of the Mg^{2+}-

stressed membranes and in line with the 'ionic strength model', the non-ionic osmolytes may have little or no activating effect in contrast to the ionic ones, which are the observations made for KdpD. Why do the amphipaths procaine and chlorpromazine fail to affect the autophosphorylation activity of KdpD in right-side-out membrane vesicles (Jung *et al*., 2000), whereas these compounds do activate *in vivo* (Sugiura *et al*., 1994)? Also here, high concentrations of magnesium may have masked an amphipath effect. If a system senses ionic strength in a mechanism in which electrostatic interactions between protein and lipids mediate the signalling, the presence of high concentrations (20 mM) of bivalent cations will have profound effects on the system. The *in vivo* activation of the KdpD system by amphipaths is only observed in mutants defective in K^+ signalling (Sugiura *et al*., 1994). If the activation of KdpD by ionic strength is mediated by the membrane environment one may not observe an amphipath effect in the wild-type system as the inhibitory K^+ signal overrides the activation by ionic osmolytes. In our opinion, the mutants, in which the K^+ and ionic strength signal are dissected (Sugiura *et al*., 1994; Jung and Altendorf, 1998), hold the most promise for complete elucidation of the sensing mechanism(s) of the KdpD sensor kinase.

Although mechanistic information on osmosensing mechanisms is most reliably obtained in artificial systems with defined protein and lipid components, such experiments and their analysis are by no means trivial. Similar to BetP from *C. glutamicum* (Rübenhagen *et al*., 2001), ProP catalyses bi-directional transport, which complicates the measurements of osmotic activation, in particular when the protein is randomly inserted in the membrane and/or the orientation after reconstitution is unknown. The ABC transporter OpuA, on the other hand, catalyses unidirectional transport and the fraction of inside-out oriented molecules does not contribute to the observed activity when ATP is only present in the lumen of the vesicles. In the case of OpuA, ATP-driven uptake of glycine betaine by right-side-in and glycine betaine-dependent ATP hydrolysis by inside-out reconstituted protein has been measured, which allowed rigorous discrimination between effects exerted at the cytoplasmic and external face of the transporter (van der Heide *et al*., 2001).

Osmotic activation of the proline transporter ProP from *E. coli (*or *Salmonella typhimurium*) has not specifically been studied as a function of osmolyte concentration and composition at the cytoplasmic face of the protein, but the reported *in vitro* data (Milner *et al*., 1988; Racher *et al*., 2001) are consistent with a mechanism in which ionic osmolyte concentration or ionic strength serve as an osmotic signal. *In vivo*, ProP from *S. typhimurium* is activated by osmotic upshift, but the system stays active even after potassium uptake has ceased (Koo *et al*., 1991). The decrease in potassium uptake is thought to reflect restoration of turgor, but the cells must still be in a state of stress as ProP is not deactivated under these conditions. As the initial response of *E. coli* and *S. typhimurium* involves the accumulation of potassium ions, it is very possible that the increased electrolyte concentration keeps the ProP system in the activated state. Taken together, the data from *in vivo* and *in vitro* studies indicate that external ionic and non-ionic osmolytes activate a range of osmosensing devices, provided the compounds do not equilibrate (cross the membrane) on the timescale of the measurements. This osmotic stress causes the cytoplasmic or liposomal volume to decrease, resulting in an increased internal osmolyte concentration. *In vivo*, not only the decrease in cell volume, but also the accumulation of potassium ions in the initial response to osmotic upshift, may lead to large increases in the cytoplasmic ionic osmolyte concentrations. As the *in vitro* studies with OpuA clearly discriminate between ionic and non-ionic osmolytes at the internal face of the protein, ionic osmolytes (or ionic strength) are postulated to serve a role as the primary signal under hyperosmotic stress.

Specific stimulus

The best documented case of an osmoregulated system that senses a specific molecule is the membrane-bound sensor kinase KdpD. Consistent with the suggestion of ionic strength as a major stimulus (see previous section), the autophosphorylation activity of KdpD increases up to at least 300 mM NaCl. In the wild-type system, this activity is specifically opposed by K^+ ions, whereas NaCl and KCl stimulate autophosphorylation activity of KdpD to the same extent in a mutant defective in K^+ sensing (Jung *et al*., 2000). Unlike the ionic strength signal, K^+ seems to be sensed by KdpD externally (Sugiura *et al*., 1994; Roe *et al*., 2000). The stimulation of KdpD activity by ionic strength serves to increase the expression of *kdpFABC* under conditions of osmotic upshift, thereby allowing *E. coli* to counteract the stress by increasing the internal osmolality through the accumulation of K^+ via the KdpFABC ATPase. The inhibitory effect of K^+ is most probably related to the specific function of the KdpD sensor kinase, that is, the induction of the *kdpFABC* operon under hyperosmotic conditions and potassium limitation. This osmoregulatory function is important when the K^+ uptake capacity via the constitutively expressed low affinity systems (TrkG, TrkH and Kup) becomes limiting, i.e. under conditions where the medium concentrations of K^+ are low.

The postulated K^+ sensing mechanism of BetP, discussed in the previous section, awaits further experimentation, i.e. the isolation of mutants and discrimination

between the effects of K^+ and Na^+. The *E. coli* mechanosensitive channel, KefA, may also be regulated by ionic osmolytes. Here, there exists a gain-of-function mutant, RQ2, resulting from a missense mutation within the *kefA* gene (McLaggan *et al.*, 2001). The phenotype is expressed as a lack of growth in media containing high K^+ concentrations and glycine betaine. Substituting Na^+ for K^+ rescues the phenotype. Presumably, this reflects an exaggeration of the wild-type KefA activity. One interpretation would be that KefA is K^+-selective. However, given the large pore size predicted from its huge conductance (1 nS), it seems more likely that there is specific ionic regulation on the periplasmic side of the protein. If so, it remains unclear if activity is stimulated by K^+, or inhibited by Na^+. Similar to MscL, KefA can be gated under patch clamp conditions by suction in the pipette. Hence, it seems likely that KefA also senses tension in the membrane. The extent of the ionic influences discussed here has yet to be explored by electrophysiological measurements.

Finally, in the pioneering studies on the osmotic regulation of the Trk K^+ transporter, Meury and colleagues (Meury et al., 1985) observed that the system, activated by osmotic upshift, was turned off gradually when the turgor was restored. These experiments have been interpreted in terms of dependence of the rate of uptake on the intracellular osmolality. In our opinion, the inhibition of uptake could be the result of feedback regulation by accumulated substrate, and the data need not necessarily be explained in terms of intracellular osmolality or ionic strength dependence. There are many more examples of transport systems that are under feedback control (often referred to as product- or trans-inhibition), but this type of regulation is not restricted to systems that are under osmotic control. The relevance of feedback regulation of transport processes in relation to osmotic stress was already discussed in a previous review (Poolman and Glaasker, 1998) and is not treated further here.

Macromolecular crowding

Cellular volume changes as a result of osmotic stress will result in changes in cytoplasmic protein concentration (macromolecular crowding), which affect the equilibrium of oligomeric enzymes and thereby their function. Although macromolecular crowding will not directly affect the function of systems embedded in the cytoplasmic membrane, membrane proteins that have a tendency to associate with soluble macromolecules may be influenced (Minton et al., 1992). This is an area of research that, in relation to osmotic stress, definitely requires further attention and, in particular, rigorous experimentation.

Osmotic upshift signal

The overview of the osmosensing mechanisms of OpuA and BetP, and the sensor kinases KdpD and EnvZ point towards intracellular ionic osmolytes as the primary signal of osmotic stress. This signal may not be sensed directly by the protein, i.e. via changes in surface hydration of the proteins or specific binding of ions by the protein; rather, the membrane in which the protein is embedded is postulated to serve as mediator. Changes in cellular ion concentrations are likely to alter specific interactions between (ionic) lipids and the proteins, thereby altering their activity.

Why would the cell use ionic osmolytes rather than intracellular osmolality (affecting protein hydration) or a specific signalling molecule (specific regulatory site on the protein)? When the medium osmolality is raised, the initial change in cytoplasmic water activity depends on the elasticity of the cell wall. Contrary to what is often thought, the cell wall of bacteria is not rigid but actually quite elastic (Csonka and Hansen, 1991; Doyle and Marquis, 1994). Consequently, even at turgors above zero, the cytoplasmic volume decreases with increasing external osmolality, and the ion (osmolyte) concentrations increase accordingly (Fig. 3). The increase in ionic strength accompanying the volume decrease is undesirable as too high concentrations of electrolytes interfere with macromolecular functioning in eubacteria as well as higher organisms (Yancey *et al.*, 1982). As well documented for *E. coli* (Higgins *et al.*, 1987; Dinnbier *et al.*, 1988; Record *et al.*,

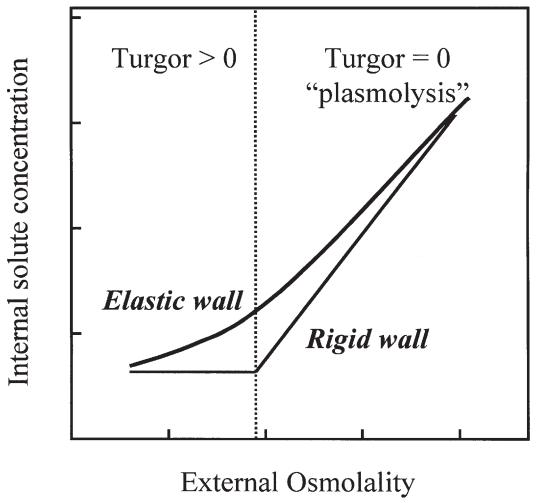

Fig. 3. Changes in cytoplasmic solute concentrations as a function of medium osmolality for cells with a rigid or elastic cell wall. Modified after Csonka and Hanson (1991). For cells with a rigid wall, the intracellular solutes concentrations change after turgor has become turgor zero. Under these 'plasmolysing' conditions, the change in intracellular solute concentration is proportional to the medium osmolality, a situation similar to that in proteoliposomes. In cells with an elastic wall, the cytoplasmic solute concentrations already increase when turgor is still positive.

1998), most eubacteria expel ionic compounds in the event the electrolyte concentration becomes too high and replace these molecules with neutral osmolytes such as glycine betaine to balance the cellular osmolality. The increase in electrolyte concentration (or ionic strength) upon a modest decrease in turgor pressure would thus represent an excellent trigger ('osmotic signal') for the activation of any osmoregulated transporter of neutral compatible solutes. Actually, it would prevent the osmotic stress from turning into 'electrolyte stress'. Why, then, is the increase in intracellular osmolality less suitable as osmotic signal? To maintain a relatively constant turgor at different external osmolalities, the cell must be able to switch on transporters and take up organic compatible osmolytes with maximal activity at different internal osmolalities. In other words, the ability of (the majority of) microorganisms to grow at their maximal rate over a wide range of medium osmolalities implies that cellular processes function optimally over a wide range of intracellular osmolalities. Finally, the cell could use the osmotic upshift-dependent change in concentration of a specific molecule as signal, but ionic strength or collective ion concentration seems to be a more general signal for osmoresponsive systems. Specific signals may be used to tune the activity or expression of a particular system as suggested by the data on the sensor kinase KdpD. Although sensing of a specific molecule, e.g. K^+ as proposed for the BetP system, will be mechanistically different from sensing the collective ion concentration (or ionic strength) with the membrane as mediator (as proposed for OpuA), the two models may be physiologically equivalent. K^+ is the dominant cation in most, if not all, cells and changes in cytoplasmic ionic strength are to a large extent due to variations in the concentrations of this inorganic cation.

Osmotic downshift signal

Upon osmotic downshift, water flows into the cell and the cytoplasmic osmolality and ion concentration decrease, turgor increases and the physiochemical properties of the membrane change as tension within the plane of the bilayer builds up. The observation that an increase in membrane tension applied by negative pressure in a patch-clamp pipette (Blount *et al*., 1999) suffices to open mechanosensitive channels indicates a sensing mechanism completely different from that of the osmotic upshift-activated transporters and sensor kinases. The molecular mechanisms, however, are far from solved. The increase in tension in the membrane following water influx could be sensed as a decrease in lateral pressure on the protein, which would facilitate the transition from the closed to the open state. The accompanying membrane thinning and the resulting hydrophobic mismatch probably also contribute to the stability of one or more of the proposed conformational states. Finally, it has been proposed, but not yet demonstrated, that hydrogen bonding may occur between the MscL protein and specific lipid headgroups, facilitating the transduction of energy from the bilayer to the protein.

Structural basis for osmosensing

A crystal structure at 0.35 nm resolution is available for MscL in a 'mostly' closed state (Chang *et al*., 1998). MscL is a homopentamer with two transmembrane α-helices per subunit (Fig. 4). It is the first transmembrane segment (TM1) that constitutes the constriction of the closed pore. Many of the amino acids of TM1 are either glycine or smaller hydrophobic residues. This structure was originally noted as a 'hydrophobic lock' (Chang *et al*., 1998; Blount and Moe, 1999). Interestingly, mutations within this

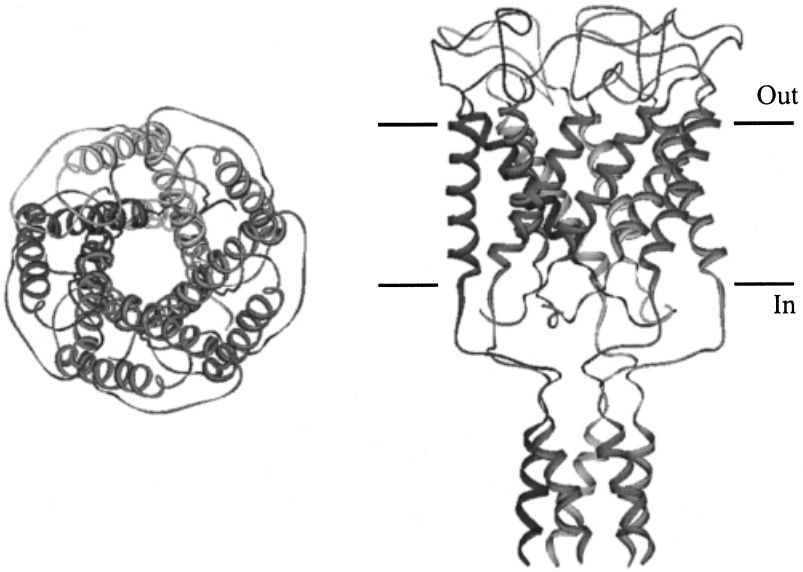

Fig. 4. The structure of the *Mycobacterium tuberculosis* MscL protein as derived using X-ray crystallography (Chang *et al*., 1998), modelled as a solid ribbon using WEBLAB VIEWER software. Note that the channel is a homopentamer with the first transmembrane segment comprising the constriction point of the (mostly) closed pore. A top view (left), looking down on the molecule from the periplasm, and side view (right), with the putative membrane boundaries, are shown.

region were repeatedly found in a random mutagenesis study in which gain-of-function mutants, with slow or no growth upon expression, were isolated (Ou *et al*., 1998). Essentially all of the mutations were to more hydrophilic residues; many of them added a charge to the molecule. A subsequent site-directed mutagenesis study showed that it is the hydrophilicity, not the size or other property of the amino acid, which correlates with the severity of the phenotype (Yoshimura *et al*., 1999). In both mutagenesis studies, the severity of the phenotype correlated with a shift of the MscL open probability to lower values of membrane tension, and with more rapid kinetics (shorter open dwell time). This latter feature suggests that the primary energy barrier between the closed and open state has been lowered. Together, these data suggest that this region of the molecule goes through a hydrophilic environment in the transition states upon channel gating. The most likely aqueous environment is the lumen of the pore. More recently, the structure of MscL has formed the basis for modelling and experimental studies, and yielded information about a potential second gate, not resolved in the crystal structure, and the open state. The five amino-terminal amphipathic α-helices have been postulated to form a cytoplasmic second gate, which is pulled apart when the transmembrane domains expand with increasing membrane tension (Sukharev *et al*., 2001a; b). In this model, the angle of the transmembrane segments increases with gating to account for the thinning of the membrane. Although this theoretical model is not yet confirmed by crystallographic data, cross-linking and other studies are beginning to support some of its predictions.

As stated in our 1998 review (Poolman and Glaasker, 1998), the structure–osmoregulation relationships in the osmotic upshift activated transporters are not readily revealed by their primary sequences. Proteins such as ProP from *E. coli* and BetP from *C. glutamicum* have terminal extensions that are likely to play a role in osmosensing (Peter *et al*., 1998; Culham *et al*., 2000), but not all homologues with the same structural features seem to be osmotically regulated (see Poolman and Glaasker, 1998). In the case of OpuA from *L. lactis*, there is no obvious domain that one could predict to have a role in osmosensing, but perhaps this is also not necessary. If OpuA senses osmotic stress via changes in ion concentration, transduced to the protein via ionic lipids, changes in electrostatic interactions between a limited number of lipid molecules and charged residues at the membrane–water interface could be sufficient for (in)activation of the transporter. The same could hold true for the ion-linked transporters ProP and BetP, but when the appropriate mutations or deletions are introduced into the terminal extensions of these proteins, changes in the activation profile can be observed; (Peter *et al*., 1998; Culham *et al*., 2000). Perhaps the isolation of mutants defective in osmoregulated uptake and/or the identification of regions in the proteins specifically interacting with anionic lipids hold the most promise for the elucidation of the structural basis for osmosensing.

In conclusion, the mechanisms of osmosensing of osmotic upshift-activated transporters and sensor kinases and osmotic downshift-activated mechanosensitive channels are clearly different. Although direct proof is available only for OpuA and MscL, the primary activation signals of both the up- and downshift activated systems seem to be transduced via the membrane. Transporters and sensor kinases appear to respond to changes in cytoplasmic concentrations of ionic osmolytes, whereas the channels sense tension in the membrane. In addition to this primary trigger, there can be fine-tuning of the activity through the sensing of more specific signals as exemplified by the K^+-specific regulation of the KdpD signal kinase. The understanding of the structural basis for osmosensing is limited, and significant information is so far confined to the MscL mechanosensitive channel protein. The transduction of the osmotic signal to the protein from the membrane offers, in principle, additional means of control via alterations in lipid composition in the process of osmotic adaptation. Such potential regulation is suggested by the *in vitro* studies with the OpuA ABC transporter, for which the ionic set-point for activation could be shifted to higher concentrations of intracellular ionic osmolytes by increasing the fraction of ionic lipids in the membrane.

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